Quantitation of human cytomegalovirus DNA in peripheral blood leukocytes of heart transplant recipients: relationship with pp65 antigenemia and with antiviral therapy

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Objective: To retrospectively determine DNA levels in blood polymorphonuclear leukocytes (PMNLs) of 21 heart transplant patients who suffered from HCMV infection and who were monitored by the antigenemia assay (pp65 test) during follow-up, by use of a quantitative competitive polymerase chain reaction (PCR) assay for human cytomegalovirus (HCMV) DNA.

Methods: Quantitation of HCMV DNA by PCR was expressed as genome equivalents (GE) per 200 000 PMNLs.

Results: Ten patients experienced symptomatic HCMV infection (five primary infections and five reactivations) with mild symptoms and received ganciclovir treatment, whereas 11 asymptomatic HCMV infections were not treated. Therapy was discontinued when a 90% reduction of the pretreatment antigenic load was achieved in a symptomless patient. The mean HCMV DNA and antigenic loads were significantly higher in symptomatic than in asymptomatic patients: $4.6\times10^5\pm4.7\times10^5$ GE and 1.1×10^4 GE ($p<0.0001$) and 390 ± 350 versus 25 ± 12 pp65-positive PMNLs ($p<0.0001$), and in primary than in secondary infections (583 ± 403 pp65-positive PMNLs versus 85 ± 111, $p=0.002$ and $5.2\times10^5\pm5.2\times10^5$ GE instead of $1.5\times10^5\pm3.2\times10^5$ GE, $p=0.02$). A single course of 14–21 days of ganciclovir caused a marked decrease of HCMV DNA and antigenemia in eight of 10 patients in whom a 90% reduction of the antigenic load correlated with a 98% DNA reduction of the pretreatment levels. In two primary infections, a 90% antigenic reduction was achieved by 21 days of ganciclovir treatment, but those data only correlated with a DNA load reduction of 28% and 60% of the pretreatment levels. Fifteen and 12 days later, respectively, the two patients relapsed and underwent a second ganciclovir course, at the end of which a 90% reduction of the antigenic load correlated with a >98% DNA drop. GCV was discontinued and the patients recovered completely. In those two patients we retrospectively found persistent high DNA levels before the second ganciclovir course, whereas the antigenic load slowly increased after an apparent reduction.

Conclusions: Our data suggest that: (1) DNA levels have the same trend as the pp65 antigen test—they are significantly higher in symptomatic and in primary HCMV-infected patients than in asymptomatic patients and those with secondary infection; (2) a 90% antigenic load reduction from the pre-treatment level may be a less reliable predictor of the efficacy of anti-HCMV therapy than DNA load, at least in primary infection, in which a much higher viral load and much more severe disease are present; and (3) a DNA load reduction of >98% of the pretreatment value is required for therapeutic success.

Key words: quantitative polymerase chain reaction, PCR, human cytomegalovirus, HCMV, heart transplantation, antigenemia, ganciclovir

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INTRODUCTION

Human cytomegalovirus (HCMV) is a well-known cause of morbidity and mortality in heart transplant (HT) patients [1–8]. With the introduction of effective anti-HCMV therapies such as ganciclovir and foscarnet [9,10], early diagnosis and pre-emptive therapy are essential for proper management of the infection. New parameters and procedures for prompt diagnosis as well as for careful monitoring of HCMV infection have been developed. Polymorphonuclear leukocytes (PMNLs) are the major HCMV carriers among white blood cells during active HCMV infection, and detection of the HCMV lower matrix phosphoprotein pp65 in PMNLs is an early marker of HCMV infection; the number of pp65-positive PMNLs correlates with the development of clinical symptoms in transplant patients [11–17]. The polymerase chain reaction (PCR) has proved to be a powerful method in diagnostic virology. Because of its high sensitivity, PCR may be used to detect HCMV in blood earlier than antigenemia, as well as for HCMV tissue localization and for monitoring anti-HCMV treatment [18–20]. However, quantitative PCR has given results of variable clinical relevance in HCMV infection [21–25]. In recent years, major advances have been made in HCMV DNA quantitation, either by PCR [26–36] or by hybridization techniques [37–39]. Published data that compared antigenemia and/or viremia and quantitative determination of HCMV DNA showed that non-PCR hybridization methods were less sensitive than PCR and antigenemia, but their results correlated with clinical symptoms better than qualitative PCR [37–39]. Quantitative PCR (qPCR) for HCMV DNA could be used to differentiate low from high viral load in blood, a high load being correlated with subsequent development of HCMV disease, and for anti-HCMV therapy monitoring. qPCR seems to be promising for monitoring HCMV infection in immunocompromised patients [26–36] but it is a highly complex technique and further studies are required to assess its clinical relevance.

In the present study, we quantitated HCMV DNA retrospectively by PCR in the PMNLs of a study group of 21 HT recipients who suffered from HCMV infection. These patients were also prospectively monitored for HCMV infection by the antigenemia assay (pp65 antigen test), and we compared the quantitative detection of pp65 antigen in the PMNLs to the DNA load detected by PCR. We concentrated on qPCR results with regard to the early identification of ongoing HCMV infection, the correlation with the development of clinical disease and monitoring antiviral treatment.

MATERIALS AND METHODS

Patients

The study group consisted of 21 of 135 patients who underwent orthotopic heart transplantation from January 1993 to January 1997 at the Cardiac Surgery Department of the University of Turin, Italy, and who had either symptomatic or asymptomatic HCMV infection in the early post-transplant period. All patients were prospectively monitored for HCMV infection on the basis of the antigenemia assay. HCMV DNA was retrospectively quantitated in the PMNLs in patients with either symptomatic or asymptomatic infection. The 21 patients of this study were the transplant patients who had had positive antigenemia and whose blood leukocytes were available for retrospective analysis.

All patients were given a triple immunosuppressive regimen of prednisone, cyclosporin and azathioprine. Rabbit antithymocyte globulin was used for the first 10 days after heart transplantation.

Human cytomegalovirus monitoring

Virologic monitoring of HCMV infection was as follows. Heparinized blood samples for the pp65 antigen test and DNA detection were collected weekly during the first month after heart transplantation and then every 15 days until the third month. From the third to the twelfth month, blood was examined monthly. In symptomatic patients, during the acute phase of HCMV infection, blood was tested twice a week.

PMNL sample preparation

PMNLs were separated by sedimentation of 3–7 mL of heparinized blood sample in a 6% dextran solution (5 vol. blood/1 vol. dextran solution). Leukocytes were centrifuged at 2500 rev/min for 10 min at room temperature, and the resulting pellet was washed in 0.8% NH_4Cl for 1–2 min to eliminate red blood cell contamination. PMNLs were then centrifuged and resuspended in 1 mL of phosphate-buffered saline (PBS), pH 7.4. Aliquots of 200 000 PMNLs were used for the pp65 antigen test and for DNA detection by PCR.

pp65 antigen test (antigenemia)

Aliquots of 200 000 PMNLs were prepared on glass slides by use of a cytocentrifuge (Cytospin 2, Shandon Southern Products, Astmoor, UK). The PMNLs were fixed in 5% formaldehyde–PBS for 10 min. After fixation, a permeabilization step with Nonidet P-40 (Sigma, St. Louis, USA) was carried out before staining with a pool of mouse monoclonal antibodies to HCMV pp65 antigen (C10-C11; Biotest, Dreieich, Germany)

for 45 min. After the first incubation, the slides were washed twice in PBS supplemented with 1% fetal calf serum and then stained with fluorescein-conjugated F(ab')2 fragment of rabbit antimouse immunoglobulins (Dako, Glostrup, Denmark). Slides were rinsed twice in PBS, and then mounted and observed under fluorescent microscopy. HCMV pp65-positive PMNLs were counted and referred to 200 000 examined cells. Different levels of antigenemia (antigenic load) were defined as follows: low, moderate and high when the numbers of pp65-positive PMNLs were respectively <5, 5–50 and >50 per 200 000 examined cells [21].

DNA extraction from leukocytes

Aliquots of 200 000 PMNLs were used for DNA detection by PCR. QIAamp spin columns were used for DNA extraction from PMNLs, according to the manufacturer's instructions (QUIAGEN GmbH, Hilden, Germany) [40].

Quantitation of HCMV DNA by PCR

We constructed a quantitative competitive PCR assay according to the method previously published by Zipeto et al [26], with some modifications. In order to generate a competitive non-homologous internal standard (IS), we used the PCR MIMICs technique for competitive PCR (PCR MIMIC Construction kit, Clontech, Palo Alto, CA, USA). The primers that we currently use for HCMV DNA detection in clinical samples are located within the translated region of the viral genome coding for the major immediate early antigen (IE1) (sequences 5′ to 3′: upstream primer AGA CCT TCA TGC AGA TCT CC, downstream primer GGT GCT CAT GCA CAT TGA TC) [41,42]. Using the PCR MIMIC technique, we generated a pair of composite primers consisting of two 40-mer oligonucleotides whose sequence at the 5′ end (nt 1–20) contained 20 bases complementary to HCMV primer sequences, and at the 3′ end (nt 21–40) 20 bases complementary to a region of a neutral-behaving heterologous DNA fragment: a 633-bp *Bam*HI/*Eco*RI fragment of the human v-*erB* gene. After the first round of PCR with the composite primers, a recombinant DNA molecule (DNA-MIMIC), including HCMV primers at its ends, was obtained that could then be amplified using only HCMV-specific primers. The size of the DNA-MIMIC molecule (340 bp) was different from that of the HCMV IE1 product of PCR (262 bp). DNA-MIMIC was cloned into competent INVaF cells (TA Cloning kit, Invitrogen, San Diego, CA, USA), as was the HCMV amplification product, in order to construct a reference external standard (ES). The resulting two plasmids were extracted from positive clones using the Pure Prep Macro Plasmid purification system (Pharmacia Biotech, Uppsala, Sweden), and following linearization they were ready for a co-amplification PCR assay. Clinical samples (PMNLs) and a known amount of the ES (number of copies from 10 to 10^6) were amplified in the presence of 10^3 copies of IS.

A reference curve for the quantitation of HCMV DNA was derived from the evaluation of the ES/IS ratio of the hybridization signals, after PCR amplification, for each point of the reference curve (number of copies from 10 to 10^6). Hybridization of the amplified products was performed with the DNA Enzyme Immunoassay (DEIA) (Sorin, Saluggia, Italy) [30]. An oligonucleotide probe within the amplified region of HCMV DNA and a probe complementary to the IS were used. HCMV DNA–IS hybridization signals (optical densities) were calculated for each sample and for each concentration of the reference HCMV standard. A logarithmic reference curve was constructed by plotting the ES/IS ratio for each standard point against its known concentration. The same ratio was calculated for each sample and used to extrapolate the copy number from the reference curve. Absolute quantitation of the samples was expressed as genome equivalents (GE)/200 000 PMNLs.

All the DNA extracted from 200 000 PMNLs in each clinical sample was subjected to DNA amplification in 100 μL PCR reaction buffer containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 20 nmol of dNTPs, 50 pmol of each primer, and 2 units of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT, USA). The PCR reaction was performed in an automated thermal cycler (DNA Thermal Cycler, Perkin-Elmer Cetus) for 40 cycles (denaturation, 94°C for 40 s, annealing, 55°C for 1 min, extension, 72°C for 3 min; plus 5 s of autoextension every cycle). Twenty microliters of each amplified sample was subjected to hybridization by the DEIA assay.

To prevent carryover of amplified DNA sequences and false-positive reactions, samples were prepared under a biosafety hood, in a room physically isolated from that in which PCR was performed. Separate sets of supplies and positive pipetting devices were dedicated to sample preparation and to set-up reactions. HCMV-infected human embryonic lung fibroblast cells were used as positive controls. Negative controls (non-infected human embryonic lung fibroblast cells), and a reagent mixture free from DNA template as '0' DNA control, were used in each run.

Diagnosis and treatment of HCMV infection

Diagnosis of HCMV infection was determined by a positive result in the pp65 antigen test (≥1 pp65-

positive PMNLs). Diagnosis of HCMV primary infection was based on virus-specific IgG seroconversion from negative to positive in a seronegative recipient (R−) before heart transplantation. In anti-HCMV seropositive patients (R+) before heart transplantation, infection was recorded as reactivation/reinfection. Infected patients were treated with 9-(1,3-dihydroxy-2-propoxymethyl) guanine (ganciclovir) at a dosage of 10 mg/kg per day intravenously for 14–21 days when symptoms of HCMV infection appeared, i.e. when fever occurred in combination with any one of the following signs: leukopenia, thrombocytopenia, rise in liver enzymes or gastroenteric signs. In our series, 10 patients experienced symptomatic HCMV systemic infection with mild symptoms. The most common clinical symptom was fever associated with leukopenia and/or thrombocytopenia. Eleven patients experienced asymptomatic HCMV infection and were not treated. None of the HCMV-infected patients required an increase of the immunosuppressive regimen due to acute rejection.

Statistical analysis

Since the pp65 and DNA values were not normally distributed, we compared the groups with the non-parametric Mann–Whitney test, and p values below 0.05 were considered to indicate statistical significance.

RESULTS

Patients

The mean time of follow-up after heart transplantation was 134±65 days (range: 40–301 days). Six of 21 patients were seronegative for HCMV before transplantation, and they received a graft from seropositive donors (D+/R−, mismatched patients), and 15 patients were seropositive for HCMV before surgery. Ten patients experienced symptomatic HCMV infection. Five of them were primary infections in D+/R− mismatched recipients. The other five were symptomatic reactivations/reinfections in seropositive recipients. Ganciclovir was administered immediately on the occurrence of mild symptoms (fever and leukopenia in all 10 cases) and was discontinued when a 90% reduction in the pretreatment antigenic value was achieved in a symptomless patient (14–21 days). A second course of ganciclovir therapy was administered to two D+/R− patients because of a high peak in the antigenic load (270 and 1000 pp65-positive PMNLs, respectively) after a first 21-day course of ganciclovir. Eleven asymptomatic HCMV infections occurred in 10 D+/R+ and in one D+/R− recipients. These patients were not treated.

Quantitation of HCMV DNA by PCR

qPCR for HCMV DNA was retrospectively performed on 103 pp65-antigen-tested samples. Seventy-two leukocyte samples were from 10 symptomatic patients, with a median number of seven samples available for each patient, while 31 were the samples available for analysis from the 11 asymptomatic transplanted patients (median number of two samples from each patient). HCMV DNA and pp65 detection were positive in 69 of 103 samples (67%), while both tests were negative in 18 samples (17%). Sixteen pp65-negative samples were positive by PCR, corresponding to the early stage of HCMV infection and after ganciclovir treatment (Table 1). The overall concordance between qPCR and antigenemia was 84.4% (87 samples).

HCMV symptomatic infected patients

Ten of 21 patients (48%) experienced a symptomatic HCMV infection. In seven patients, HCMV infection was detected within 50 days from heart transplantation, and in three on days 53, 56 and 143 after surgery. Table 2 shows HCMV infection monitored by the pp65 antigen test and HCMV DNA by qPCR. After heart transplantation, the mean first day of HCMV detection in PMNLs was 45±36 (range 20–143) by the pp65 antigen test and 41±32 (range 20–126) by PCR. HCMV DNA was detected by PCR a mean of 12 days prior to antigenemia (range 7–17 days) in three patients. In the other seven patients, the two tests were positive simultaneously. Before therapy, the mean antigenic load was 390±350 pp65-positive PMNLs, and HCMV DNA mean levels were $4.6\times10^5\pm4.7\times10^5$ GE (range 5×10^4 to 10^6). These data differed significantly from those obtained in asymptomatic patients, in whom the mean antigenic load was 25±12 pp65-positive PMNLs ($p<0.0001$) and the mean DNA level was $1.1\times10^4\pm2.3\times10^4$ GE ($p<0.0001$), as shown in Table 3. A single course of ganciclovir therapy caused a marked decrease of HCMV DNA and antigenemia but significant differences were observed among patients. Eight of 10 patients (three D+/R− and five D+/R+ transplants) showed a prompt response to a single 14 to

Table 1 Comparison between quantitative PCR (qPCR) and pp65 antigen test: number of positive and negative samples (PMNLs) obtained with the two tests

		qPCR		
		+	−	Total
pp65	+	69	0	69
	−	16	18	34
	Total	85	18	103

Table 2 Monitoring of HCMV symptomatic infections and antiviral treatment in 10 heart transplant patients with the pp65 antigen test and quantitative PCR for HCMV DNA detection

		Day of first detection from heart transplantation		Levels at therapy		Levels at the end of therapy		
Patients	D/R status	pp65	HCMV DNA	pp65[a]	HCMV DNA[b]	pp65[a]	HCMV DNA[b]	Ganciclovir: days of therapy[c]
1	D+/R−	36	36	96	70000	0	<10	14
3	D+/R+	24	24	80	10^5	5	1000	14
3	D+/R+	32	21	420	50000	0	<10	14
4	D+/R−	143	126	300	10^6	0	20	14
5	D+/R+	20	20	125	150000	0	<10	14
6	D+/R+	39	39	80	10^6	1	100	14
7	D+/R+	34	34	95	90000	7	4000	14
8	D+/R−	53	53	1000	10^6	4	3000	21
9	D+/R−	29	22	760	70000	55	50000	21
								1st course
				1000	50000	0	<10	21
								2nd course
10	D+/R−	36	36	450	10^6	40	4×10^5	21
								1st course
				270	10^6	1	100	21
								2nd course

[a]Number of pp65-positive PMNLs/200000 PMNLs. [b]Number of genome equivalents/200000 PMNLs. [c]10 mg/kg per day.

Table 3 Monitoring of HCMV asymptomatic infections in 11 heart transplant patients with the pp65 antigen test and quantitative PCR for HCMV DNA detection

		Day of first detection from heart transplantation		Maximum level reached during follow-up		
Patients	D/R status	pp65	HCMV DNA	pp65[a]	HCMV DNA[b]	HCMV DNA persistence (days from first detection)
1	D+/R+	41	23	30	80000	NA[c]
2	D+/R+	18	18	35	4000	NA[c]
3	D+/R+	100	100	21	1000	89
4	D+/R−	188	188	20	200	30
5	D+/R+	36	17	20	3000	70
6	D+/R+	40	40	20	1000	37
7	D+/R+	45	45	20	2000	33
8	D+/R+	32	32	6	20000	30
9	D+/R+	33	33	15	100	31
10	D+/R+	30	30	40	3000	29
11	D+/R+	47	31	50	10000	162

[a]Number of pp65-positive PMNLs/200000 PMNLs. [b]Number of genome equivalents/200000 PMNLs. [c]Not available because the follow-up is still ongoing and HCMV DNA is still detectable in blood after 105 and 57 days respectively.

21-day course of therapy (Table 2, patients 1–8). During treatment, their mean antigenic and DNA loads were 274 ± 318 pp65-positive PMNLs and $4.3\times10^5\pm4.7\times10^5$ GE, respectively. The mean time to a 90% antigen load reduction during therapy was 13 ± 6 days. At the end of a single ganciclovir course, in three patients antigenemia and DNA were negative, in one patient antigenemia was absent but the DNA was still detectable (Table 2, patient 4), and in four patients antigenemia and DNA were both positive (mean antigenic and DNA load: four pp65-positive cells and 1600 GE, i.e. a >98% reduction of antigenic and DNA pretreatment values). No HCMV relapse was experienced by the eight recipients during their follow-up.

Before therapy, in two D+/R− patients (Table 2, patients 9 and 10) antigenemia was very high (760 and 450, respectively, pp65-positive cells) and their pretreatment DNA peaks were 5×10^4 and 1×10^6 GE, respectively. They received a first 21-day course of

ganciclovir, at the end of which a 90% reduction of the pretreatment antigenic load was shown (55 and 40 pp65-positive PMNLs, respectively), but we found that only 28% (patient 9) and 60% (patient 10) reductions of DNA pretreatment values were achieved, corresponding to 5×10^4 (patient 9) and to 4×10^5 GE (patient 10) in 200,000 PMNLs. As the patients were symptomless and a 90% reduction in antigenic load had been achieved, ganciclovir was discontinued. After 15 and 12 days, respectively, the two patients experienced an asymptomatic relapse of HCMV with a high peak of antigenemia (1000 and 270 pp65-positive cells)

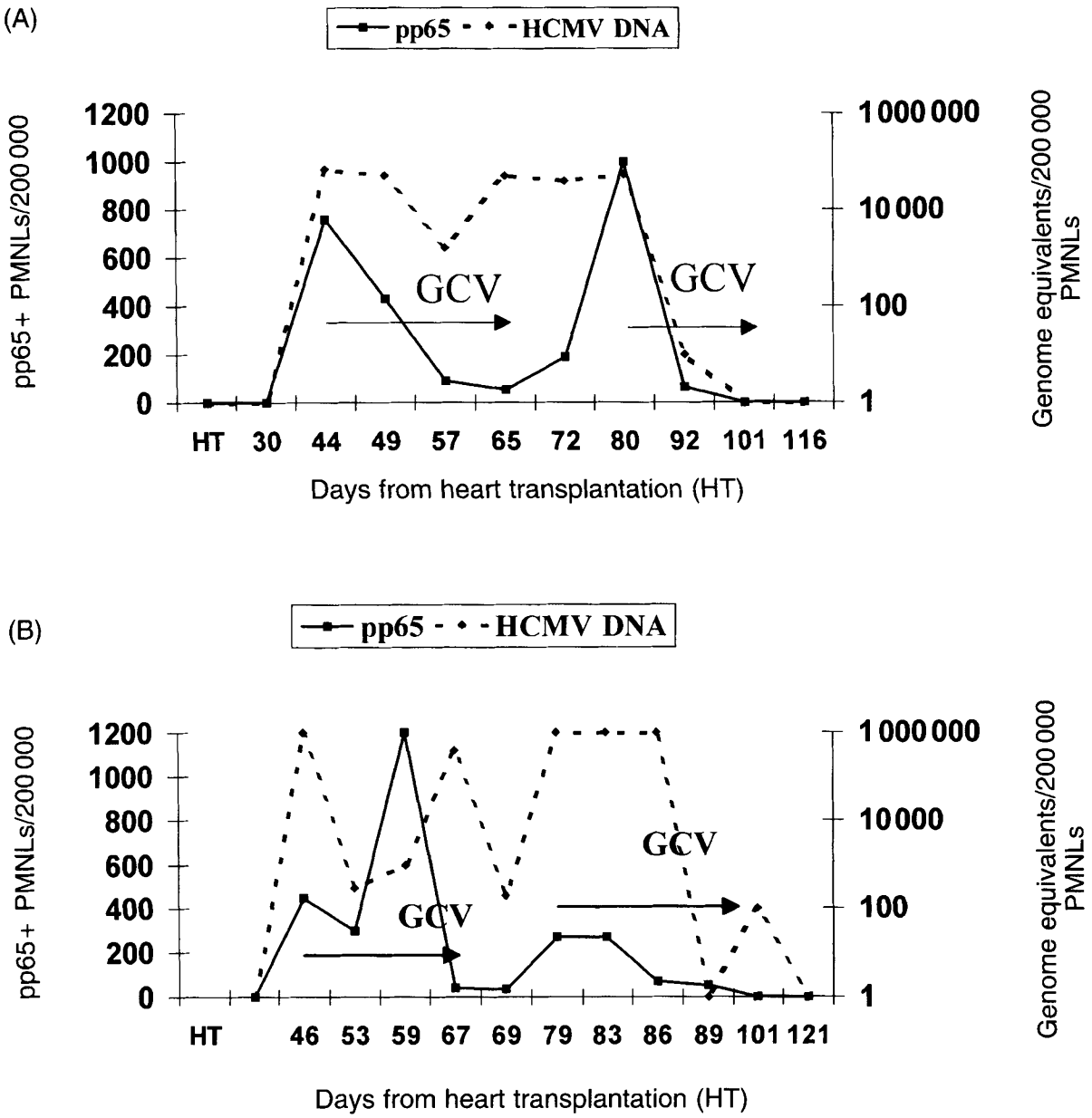

Figure 1 Graphical description of two HCMV symptomatic infections in two D+/R− patients: (a) patient 9 of Table 2; (b) patient 10 of Table 2. The two recipients relapsed after the first 21-day ganciclovir (GCV) course, which was discontinued because antigenemia dropped by >90% of pretreatment values (55 and 35 pp65-positive PMNLs, respectively). As can be seen, in the period in which antiviral therapy was not administered (15 days in patient 9, and 12 days in patient 10), HCMV DNA peaks were persistently positive, while antigenemia was slowly increasing after an apparent 90% reduction. The second antiviral course was administered when antigenemia reached 1000 (patient 9) and 270 (patient 10) pp65 PMNLs/200 000.

Table 4 Comparison between the pp65 antigen test and quantitative PCR (qPCR) in symptomatic and asymptomatic HCMV-infected recipients before heart transplantation

Parameters	HCMV symptomatic infection (n=10)	HCMV asymptomatic infection (n=11)	Statistical evaluation
pp65: day of first detection from HT	45±36	55±49	NS
pp65: antigenic load[a]	390±350	25±12	$p<0.0001$[c]
qPCR: day of first detection from HT	41±32	51±51	NS
DNA peak[b]	$4.6\times10^5\pm4.7\times10^5$	$1.1\times10^4\pm2.3\times10^4$	$p<0.0001$[d]

[a]Number of pp65-positive PMNLs/200 000 PMNLs. [b]Number of genome equivalents/200 000 PMNLs.
[c] T=165, Mann–Whitney test. [d] T=162, Mann–Whitney test.

corresponding to 5×10^4 and 1×10^6 GE in PMNLs (Table 2). They received a second 21-day course of ganciclovir. A 90% reduction in antigenic load took place at days 12 and 10 during therapy (pp65 was undetectable in patient 9, but one pp65-positive PMNL was still observed in patient 10). DNA was undetectable in patient 9, and 100 GEs were still present in patient 10. During the period in which ganciclovir was discontinued, we retrospectively found persistent high HCMV DNA levels (4×10^4 GE and 1×10^6 GE in patients 9 and 10, respectively), with increasing levels of antigenemia, following the first apparent 90% reduction. After the second 21-day ganciclovir course, the two patients recovered completely and did not experience any further HCMV relapse. Figure 1 shows the antigenic and HCMV DNA levels during monitoring in patients 9 (Figure 1a) and 10 (Figure 1b).

HCMV asymptomatic infected patients

Eleven out of 21 patients (52%) experienced asymptomatic HCMV infection and were not treated. Ten were D+/R+ and one was D+/R−. The mean first day of HCMV detection in PMNLs after heart transplantation was 55±49 (range 18–188) by the pp65 antigen test and 51±51 (range 17–188) by PCR. HCMV DNA was detected by PCR prior to antigenemia in three patients (a mean of 17 days, range 16–19 days). In eight patients the two tests were positive simultaneously. Table 3 shows the antigenic load and HCMV DNA levels in asymptomatic patients. Two parameters differed significantly between asymptomatic and symptomatic HCMV-infected patients (Table 4). In asymptomatic patients, the highest antigenic load and HCMV DNA maximum levels were significantly lower than in symptomatic recipients: 25±12 pp65-positive PMNLs (range 6–50, $p<0.0001$) and $1.1\times10^4\pm2.3\times10^4$ GE (range 100 to 8×10^4, $p<0.0001$). The mean times of HCMV antigenemia and DNA clearance from blood were 50±38 days (range 15–136) and 57±45 days (range 29–162), from the first detection, respectively.

Between primary and secondary infections, we found that the highest level of pp65-positive PMNLs and the highest DNA load, both parameters assessed before therapy in symptomatic patients, were significantly different. The pp65 and the DNA values were higher in primary than in secondary infections: 583±403 pp65-positive cells versus 85±111 (p=0.002), and $5.2\times10^5\pm5.2\times10^5$ GE versus $1.5\times10^5\pm3.2\times10^5$ GE (p=0.02).

DISCUSSION

Our data show that the quantitative determination of HCMV DNA by PCR has the same trend as the antigenic load (pp65 antigen test detection), in symptomatic and asymptomatic and in primary as well as secondary HCMV infection. HCMV DNA peaks were significantly higher in symptomatic than in asymptomatic infections, and in primary versus secondary infection, as was the antigenic load. These data completely agree with previous published observations on quantitative PCR results for HCMV DNA in bone marrow transplants and in AIDS patients [25,27,28] as well as in organ transplants [26]. In our HT patients we found that a DNA load of $1.1\times10^4\pm2.3\times10^4$ GE, corresponding to 25±12 pp65-positive PMNLs, remained asymptomatic in 10 out of 15 D+/R+ and in only one out of six D+/R− transplant patients. These data agree with published observations that the immune system in D+/R+ patients can more easily cope with HCMV infection than that in D+/R− patients [43].

The effect of antiviral treatment was evaluated in 10 HT symptomatic patients, and important variability was present. In eight of them we found a correlation between antigenemia, HCMV DNA levels and a complete response to anti-HCMV therapy. These were five D+/R+ and three D+/R− recipients in whom a response to antiviral therapy was associated with a

persistent decrease (five patients) or the disappearance (three patients) of either pp65 antigen or DNA from blood. In these patients a 90% reduction in antigenic load was achieved in 14–21 days of therapy and corresponded to a >98% reduction in DNA pretreatment values. During therapy in these patients, decreasing levels in the antigenic load were associated with decreasing levels of viral DNA. After a single ganciclovir course, low levels of antigenemia and DNA (four pp65-positive cells and 1600 GE) were completely controlled without HCMV relapse in five patients until complete disappearance of the two markers. In these patients we observed a good correlation between a 90% reduction of antigenemia, a >98% reduction of HCMV DNA and a complete response to anti-HCMV therapy.

In two out of five primary and symptomatic infected recipients, we did not achieve a 98% reduction in DNA load with a single 21-day ganciclovir course, even though we showed a 90% drop in the antigenic load. At that time, in these two patients the DNA levels only decreased to 72% and 40% of the pretreatment levels, suggesting retrospectively a lack of or a delay in response and the need for continuing antiviral therapy. After the first ganciclovir course, the mean pp65 antigen and DNA load were 47 positive PMNLs and 2.2×10^5 GE, respectively. The two patients relapsed and a second ganciclovir course was administered. During the second course, we obtained a >90% reduction of the antigenic load over a range of 10–14 days; at that time, DNA was undetectable in one patient, and in the other one a >98% reduction of the pretreatment value was achieved. We now think that ganciclovir should not have been discontinued in these two patients because antigenemia was still positive: even though it was at a moderate level, it indicated that a significant viral load could still be present in such patients.

In our experience, a >98% reduction of DNA pretreatment values is required for a complete and sustained response to anti-HCMV therapy. Our DNA quantitation system suggests that more prolonged ganciclovir therapy should be administered to primarily infected patients in order to achieve a complete response to ganciclovir and to avoid relapses. Furthermore, a 90% reduction in pretreatment antigenic levels may be a less reliable predictor of the efficacy of anti-HCMV therapy than DNA load, at least in primary infection, in which a much higher viral load and more severe disease are present. In these infections, a 90% antigenic load reduction of pretreatment values is likely to be insufficient for complete recovery, avoiding relapses in HCMV infection, because of the very high viremia. Thus, patients with primary symptomatic infection should be treated until pp65 antigen is low (<10 positive cells) or negative, at which point DNA load should also be very low (>98% reduction from the pretreatment level) or absent, as shown by the present data.

These results are in agreement with published data on anti-HCMV therapy monitored by qPCR in bone marrow transplant recipients and in AIDS patients [28,29,31,32,34–36] Those studies also showed how pre-emptive therapy can be started, based on HCMV DNA levels, and that PCR is the method of choice for early HCMV detection in those high-risk populations. Quantitation of HCMV DNA seems to be a more appropriate way of monitoring HCMV infection and also for deciding antiviral strategies in HT recipients, with particular regard to D+/R− mismatched patients who are at higher risk of symptomatic HCMV infection.

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